UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	5.1158 Div. I
First Named Inventor or Application Identifier	Hideki Kawasaki
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. [ ] Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)

2. [ ] Applicant claims small entity status.
See 37 CFR 1.27.

3. [X] Specification *Total Pages* 46

4. [X] Drawing(s) *(35 USC 113)* *Total Sheets* 2

5. [X] Oath or Declaration *Total Pages* 2

   a. [ ] Newly executed (original or copy)

   b. [X] Copy from a prior application (37 CFR 1.63(d))
   (for continuation/divisional with Box 17 completed)
   [Note Box 6 below]

      i. [ ] DELETION OF INVENTOR(S)
      Signed Statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

6. [X] Application Data Sheet. See 37 CFR 1.76

ADDRESS TO:

Commissioner for Patents
Box Patent Application
Washington, DC 20231

7. [ ] CD-ROM or CD-R in duplicate, large table or Computer Program *(Appendix)*

8. [ ] Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)

   a. [ ] Computer Readable Form (CRF)

   b. Specification Sequence Listing on:

      i. [ ] CD-ROM or CD-R (2 copies); or

      ii. [ ] paper

   c. [ ] Statements verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

9. [ ] Assignment Papers (cover sheet & document(s))

10. [ ] 37 CFR 3.73(b) Statement *(when there is an assignee)* [ ] Power of Attorney

11. [ ] English Translation Document *(if applicable)*

12. [X] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations

13. [X] Preliminary Amendment

14. [X] Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)

15. [ ] Certified Copy of Priority Document(s)
(if foreign priority is claimed)

16. [ ] Other: _____

17. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*

[ ] Continuation [X] Divisional [ ] Continuation-in-part (CIP) of prior application No. 08 / 894,344

Prior application information: *Examiner* Peter Tung, Ph.D. *Group/Art Unit:* 1652

For CONTINUATION OR DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

18. CORRESPONDENCE ADDRESS

[X] Customer Number or Bar Code Label 05514 (Insert Customer No. or Attach bar code label here) or [ ] Correspondence address below

NAME					
Address					
City		State		Zip Code	
Country		Telephone		Fax	

+

CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS (37 CFR 1.16(c))	23 -20 =	0	X $ 18.00 =	$54.00
	INDEPENDENT CLAIMS (37 CFR 1.16(b))	3 -3 =	0	X $80.00 =	$0.00
	MULTIPLE DEPENDENT CLAIMS (if applicable) (37 CFR 1.16(d))			$270.00 =	$270.00
				BASIC FEE (37 CFR 1.16(a))	$710.00
			Total of above Calculations =		$1034.00
	Reduction by 50% for filing by small entity (Note 37 CFR 1.9, 1.27, 1.28).				
				TOTAL =	$1034.00

19. Small entity status

a. [ ] A small entity statement is enclosed

b. [ ] A small entity statement was filed in the prior nonprovisional application and such status is still proper and desired.

c. [ ] Is no longer claimed.

20. [X] A check in the amount of $ 1034.00 to cover the filing fee is enclosed.

21. [ ] A check in the amount of $ _____ to cover the recordal fee is enclosed.

22. The Commissioner is hereby authorized to credit overpayments or charge the following fees to Deposit Account No. 06-1205:

a. [X] Fees required under 37 CFR 1.16.

b. [X] Fees required under 37 CFR 1.17.

c. [ ] Fees required under 37 CFR 1.18.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED	
NAME	Lawrence S. Perry Registration No. 31,865
SIGNATURE	
DATE	October 3, 2000

NY_MAIN 114365 v 1

INVENTOR INFORMATION

Inventor One Given Name:: Hideki
Family Name:: Kawasaki
Postal Address Line One:: 403-1-303, Arakawaoki-Nishi-ku, 2-chome
City:: Tsuchiura-shi
State or Province:: Ibaraki 300-11
Country:: Japan

Inventor Two Given Name:: Masaya
Family Name:: Tokai
Postal Address Line One:: 3-5-9-209, Wakabayashi
City:: Setagaya-ku
State or Province:: Tokyo 154
Country:: Japan

Inventor Three Given Name:: Yasuhiro
Family Name:: Kikuchi
Postal Address Line One:: 2-11-2-3-3, Matushiro
City:: Tsukuba-shi
State or Province:: Ibaraki 305
Country:: Japan

Inventor Four Given Name:: Kozo
Family Name:: Ouchi
Postal Address Line One:: 2-2-9, Sakuradai
City:: Hasuda-shi
State or Province:: Saitama 349-01
Country:: Japan

CORRESPONDENCE INFORMATION

Correspondence Customer Number:: 05514
Fax:: (212) 218-2200

APPLICATION INFORMATION

Title Line One:: NOVEL YEAST GENE

Total Drawing Sheets:: 2
Formal Drawings?:: Yes
Application Type:: Utility
Docket Number:: 5.1158 DIV I
Secrecy Order in Parent Appl.?:: No

REPRESENTATIVE INFORMATION

Representative Customer Number:: 5514

PRIOR FOREIGN APPLICATIONS

Foreign Application One:: 343700/95
Filing Date:: December 12, 1995
Country:: Japan
Priority Claimed:: Yes

5.1158 DIV. I

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	:	Examiner: Peter Tung, Ph.D.
HIDEKI KAWASAKI, ET AL.	)	
	:	Group Art Unit: 1652
Application No.: (Divisional	)	
of Serial No. 08/894,344	:	
filed August 15, 1997)	)	
	:	
Filed: Currently herewith	)	
	:	
For: NOVEL YEAST GENE	)	October 3, 2000

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to action on the merits, please amend the above-identified application as follows:

IN THE TITLE:

Please amend the Title to read: --PROTEIN COMPLEMENTING YEAST LOW TEMPERATURE-SENSITIVITY FERMENTABILITY--.

IN THE SPECIFICATION:

Page 1, line 3, add --This application is a division of application No. 08/894,344 filed August 15, 1997--.

Page 10, line 14, change "lowered" to --improved--.

IN THE CLAIMS:

Please cancel Claim 1 and 2.

Please amend claims 6 and 8-10 as follows:

Claim 6, line 1, delete "or 5".

Claim 8, lines 1-2, change "any one of Claims 4-7" to --Claim 4--.

Claim 9, line 2, change "any one of claims 4-7" to --Claim 4--.

Claim 10, line 2, change "any one of claims 4-7" to --Claim 4--.

Please add the following new Claims 11-14:

--11. The yeast according to Claim 5, wherein the sequence at positions 4388 through 7885 in the nucleotide sequence represented by SEQ ID NO: 1 is disrupted.

12. Dough containing the yeast according to Claim 11.

13. A process for making bread which comprises adding the yeast according to Claim 11 to dough.

14. A process for producing ethanol which comprises culturing the yeast according to Claim 11 in a medium, allowing ethanol to accumulate in the culture, and recovering ethanol from the culture.--

REMARKS

The specification has been amended to correct an inadvertent clerical error. The claims have been amended and new Claims 11-14 added to maintain their dependency in conformity with accepted U.S. practice. No new matter has been added.

Entry hereof is earnestly solicited.

Applicants undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,

Attorney for Applicant
Lawrence S. Perry
Registration No. 31,865

FITZPATRICK, CELLA, HARPER & SCINTO
30 Rockefeller Plaza
New York, New York 10112-3801
Facsimile: (212) 218-2200

LSP\ac

NY_MAIN 114379 v 1

SPECIFICATION
NOVEL YEAST GENE

Technical Field

The present invention relates to a process for making bread with refrigerated dough and a process for producing ethanol.

Background Art

Recently, in the bread manufacturing industry, a method for making bread with refrigerated dough has been widely used with the purpose of saving labor in the bread making process and meeting diverse needs of consumers. In this method, partially fermented dough is stored at a low temperature in a refrigerator and then is subjected to fermentation, proofing and baking to make bread. Such a method is usually carried out by the use of refrigeration-resistant yeast, that is, yeast which is capable of controlling fermentation during the storage of dough at a low temperature and allowing normal fermentation at temperatures for fermentation and proofing to raise the dough.

As for the breeding of refrigeration-resistant yeast, there are known methods in which yeast strains of wild type are conferred with the mutation exhibiting low-temperature-sensitive fermentability by artificial mutagenesis [e.g., Japanese Published Examined Patent Application No. 71474/95, Japanese Published Unexamined Patent Application No. 213277/95, Japanese Published Unexamined Patent Application No. 79767/95, and Appl. Environ. Microbiol., 61, 639-642 (1995)]. The yeast strains conferred with the mutation exhibiting low-temperature-sensitive fermentability are used as refrigeration-resistant yeast or as parent strains for breeding refrigeration-resistant yeast.

However, such mutagenesis induces mutation at random

and thus may possibly confer the yeast with mutation relating to the basic properties of fermentation such as dough raising, in addition to the low-temperature-sensitivity mutation.

It is also known to confer baker's yeast or brewer's yeast with favorable properties such as flocculation [The 23rd European Brewery Conv. Proc., 297-304 (1991)] and flavor [Curr. Genet., 20, 453-456 (1991)] by using gene manipulation techniques.

However, a gene relating to the low-temperature-sensitivity of fermentability or a method for breeding refrigeration-resistant yeast by gene manipulation is not known.

Ethanol is produced by fermentation of sugar materials (e.g. molasses) or starch materials (e.g. corn and potato) as carbon sources. Fermentation can be generally carried out at a temperature of 30 to 43°C. Usually, the fermentation temperature is adjusted to 30 to 35°C by cooling in order to avoid the death, insufficient growth, or decrease in fermentability of yeast caused by the rise of temperature. However, in the summer months, cooling is often insufficient, thereby causing the rise of culturing temperature to 35 to 38°C in the course of alcohol fermentation. Thus, alcohol fermentation is usually carried out with further cooling to prevent the rise of temperature due to fermentation heat. A need exists for temperature-resistant yeast which is useful for saving cost for cooling in such process.

As for the breeding of thermotolerant yeast, there have been reports on a method in which mitochondria relating to thermotolerance is introduced [Juan Jimenez, et al.: Curr. Genet., 13, 461-469 (1988)] and a method in which heat shock protein HSP104 is expressed at a high level [Susan Lindquist, et al.: Proc. Natl. Acad. Sci. USA, 93, 5301-5306 (1996)]. However, application of these methods to alcohol fermentation has not been studied. Further, it is known that the heat-resistance of yeast is

improved by heat treatment at temperatures which are not fatal to the yeast [B.G. Hall: J. Bacteriol., 156, 1363 (1983)], but this effect is not lasting, and it is difficult to apply this method to alcohol fermentation.

Disclosure of the Invention

The present invention relates to a protein having the amino acid sequence represented by SEQ ID NO: 1, or a protein being capable of complementing the mutation exhibiting low-temperature-sensitive fermentability and having an amino acid sequence wherein one or more amino acid residues are added, deleted or substituted in the amino acid sequence represented by SEQ ID NO: 1; a gene which encodes said protein; and a gene which comprises DNA having the nucleotide sequence represented by SEQ ID NO: 1, or comprises DNA being capable of complementing the mutation exhibiting low-temperature-sensitive fermentability and having a nucleotide sequence wherein one or more DNAs are added, deleted or substituted in the nucleotide sequence represented by SEQ ID NO: 1. The present invention also relates to yeast belonging to the genus *Saccharomyces* and having low-temperature-sensitive fermentability which is characterized in that the above-mentioned gene on the chromosome is inactivated; dough containing said yeast; a process for making bread which comprises adding said yeast to dough; and a process for producing ethanol which comprises culturing said yeast in a medium, allowing ethanol to accumulate in the culture, and recovering ethanol from the culture.

The expression "having low-temperature-sensitive fermentability" as used herein means the property of having substantially no fermentability at temperatures for low temperature storage and having normal fermentability at temperatures for fermentation and proofing after the low temperature storage. For instance, in the case of baker's yeast, it means the property of having substantially no dough-raising ability at 5°C and having normal dough-

raising ability at 20 to 40°C after the storage under refrigeration at 5°C for 1 to 7 days, and in the case of brewer's yeast, it means the property of having substantially no alcohol fermentability at 5°C and having normal alcohol fermentability at 20 to 40°C after the storage under refrigeration at 5°C for 1 to 7 days.

Isolation of a gene which complements the mutation exhibiting low-temperature-sensitive fermentability, determination of the DNA sequence of said gene, and inactivation of said gene can be carried out by using basic techniques for genetic engineering and biological engineering according to the descriptions in commercially available experiment manuals, e.g. Gene Manual, Kodansha Co., Ltd.; Methods for Experiments in Gene Manipulation, edited by Yasutaka Takagi, Kodansha Co., Ltd.; Molecular Cloning, Cold Spring Harbor Laboratory (1982); Molecular Cloning, 2nd ed., Cold Spring Harbor Laboratory (1989); Methods in Enzymology, 194 (1991); and Gene Experiments Using Yeasts (an extra number of Experimental Medicine), Yodosha Co., Ltd. (1994).

The gene which complements the mutation exhibiting low-temperature-sensitive fermentability according to the present invention (hereinafter referred to as the gene complementing low-temperature-sensitivity) can be isolated, for example, as the gene complementing the low-temperature-sensitivity of fermentability of Saccharomyces cerevisiae RZT-3 (FERM BP-3871) (hereinafter referred to as RZT-3 strain) described in Japanese Published Unexamined Patent Application No. 336872/93. That is, the gene complementing low-temperature-sensitivity can be isolated by transforming RZT-3 strain with the DNA library of the yeast carrying the gene complementing low-temperature-sensitivity, and obtaining DNA from the strain of which the mutation exhibiting low-temperature-sensitive fermentability is complemented.

The DNA library of the yeast carrying the gene complementing low-temperature-sensitivity can be prepared

by cleaving the chromosomal DNA of yeast carrying a gene of wild type, e.g. Saccharomyces cerevisiae X2180-1B (hereinafter referred to as X2180-1B strain) with a restriction enzyme, and ligating each of the obtained DNA fragments with a vector capable of being maintained in yeast.

Any restriction enzymes which can cleave the chromosomal DNA can be used in the above process. Preferably, those which give DNA fragments of 20 Kbp or less are used. The chromosomal DNA may be completely digested or partially digested with the restriction enzyme.

Examples of the vectors capable of being maintained in yeast are YCp vectors, YEp vectors, YRp vectors, YIp vectors, and YAC (yeast artificial chromosome) vectors.

The transformation of RZT-3 strain with the DNA library can be carried out according to the methods generally used in genetic engineering and biological engineering such as the spheroplast method [e.g. Proc. Natl. Acad. Sci. USA, 75, 1929-1933 (1978)], the lithium acetate method [e.g. J. Bacteriol, 153, 163-168 (1983)], and the electroporation method [e.g. Methods in Enzymology, 194, 182-187 (1991)].

The complementation of the mutation exhibiting low-temperature-sensitive fermentability can be confirmed by examining the transformed yeast for the growth at a low temperature or the fermentability at a low temperature [Appl. Environ. Microbiol., 61, 639-642 (1995)]. The examination on fermentability at a low temperature can be carried out, for example, by the pigment agar layer method described below. In this method, the test strain is cultured at 30°C on YPG agar medium (1% yeast extract, 2% peptone, 3% glycerol, and 2% agar) to form colonies. Then, a pigment agar (0.5% yeast extract, 1% peptone, 10% sucrose, 0.02% Bromocresol Purple, and 1% agar, pH 7.5) is layered over the medium, and the plate is kept at a low temperature (e.g. 5°C). Bromocresol Purple is a pH

indicator, and the pigment agar assumes a purple color when being layered. Fermentation of the yeast lowers the pH of the medium around the colony, thereby causing the change of the color of that area from purple to yellow. Accordingly, a strain showing the color change to yellow around the colony while the layered plate is kept at a low temperature can be selected as a strain having fermentability at a low temperature.

Recovery of a plasmid from the yeast and transformation of Escherichia coli using the plasmid can be carried out according to the methods generally used in genetic engineering. For example, the plasmid can be recovered by the method described in Gene Experiments Using Yeasts (an extra number of Experimental Medicine), Yodosha Co., Ltd. (1994), and the transformation can be carried out by the method described in Molecular Cloning, 2nd ed., Cold Spring Harbor Laboratory (1989).

The nucleotide sequence of the gene complementing low-temperature-sensitivity can be determined by the methods generally used in genetic engineering such as the Maxam-Gilbert method and the dideoxy method.

The polypeptide encoded by the gene complementing low-temperature-sensitivity can be readily obtained by using current knowledge of molecular genetics. If necessary, analysis using computers can be made [e.g. Cell Technology, 14, 577-588 (1995)]. It is possible to use the polypeptide encoded by the gene complementing low-temperature-sensitivity as an inhibitor to the low-temperature-sensitivity of fermentability in the yeast having low-temperature-sensitive fermentability.

The present invention has clarified the nucleotide sequence of the gene complementing low-temperature-sensitivity and the amino acid sequence of the polypeptide encoded by the gene, and thereby has enabled disruption of the gene complementing low-temperature-sensitivity, regulation of expression or alteration of expression level of the gene complementing low-temperature-sensitivity by

modification of the promoter, expression of various genes by the use of the promoter of the gene complementing low-temperature-sensitivity, preparation of a fused gene in which the gene complementing low-temperature-sensitivity is fused with another gene as well as a fused polypeptide, and the like. These manipulations can be carried out by using, for example, the methods described in Methods in Enzymology, 194, 594-597 (1991).

The methods for inactivating the gene complementing low-temperature-sensitivity in yeast are described below.

The term inactivation of the gene as used herein refers to the lowering or loss of functions inherent in the gene or the polypeptide encoded by the gene induced by various techniques for genetic engineering or biological engineering; for example, gene disruption [e.g. Methods in Enzymology, 194, 281-301 (1991)], introduction of a movable genetic element into the gene [e.g. Methods in Enzymology, 194, 342-361 (1991)], introduction and expression of the antisense gene [e.g. Japanese Published Examined Patent Application No. 40943/95, and The 23rd European Brewery Conv. Proc., 297-304 (1991)], introduction of DNA relating to silencing to the vicinity of the gene [e.g. Cell, 75, 531-541 (1993)], and treatment of the polypeptide encoded by the gene with an antibody [e.g. European J. Biochem., 231, 329-336 (1995)].

For the inactivation of the gene complementing low-temperature-sensitivity, any yeast which belongs to the genus Saccharomyces, preferably Saccharomyces cerevisiae, can be used. That is, various kinds of yeasts such as baker's yeast, sake yeast, wine yeast, beer yeast, miso and soy sauce yeast, and ethanol-producing yeast belonging to the genus Saccharomyces can be used.

The disruption of the gene complementing low-temperature-sensitivity means a process which comprises introducing into yeast cells DNA which has a nucleotide sequence homologous to that of the gene complementing low-

temperature-sensitivity but is incapable of acting as the gene complementing low-temperature-sensitivity due to a mutation such as addition, deletion or substitution, to induce homologous recombination, and thereby incorporating this mutation into the gene on the genome.

The DNA used for the gene disruption can be prepared, for example, by a method which comprises cleavage of the gene complementing low-temperature-sensitivity with restriction enzymes to add, delete or substitute DNAs, and a method which comprises extracellular mutation (in vitro mutagenesis) of the gene complementing low-temperature-sensitivity. For the addition and substitution of DNAs, a method can be used in which the marker gene is inserted.

The disruption of the gene complementing low-temperature-sensitivity can be effected by disruption of any of the promoter region, open reading frame region, and terminator region of the gene, or combinations of such regions. The gene complementing low-temperature-sensitivity can also be disrupted by deleting the entire gene.

The disruption of the gene complementing low-temperature-sensitivity can be carried out, for example, by transforming yeast with a plasmid for the disruption of the gene complementing low-temperature-sensitivity of the yeast or a fragment of the plasmid to induce homologous recombination of a DNA fragment carried on the transforming plasmid or its fragment with the gene on the genome of the yeast. The plasmid for the disruption of the gene complementing low-temperature-sensitivity or its fragment must have homology to the gene complementing low-temperature-sensitivity on the genome of the yeast in a degree sufficient for the induction of homologous recombination. A DNA fragment can be examined for the capability of inducing homologous recombination by introducing the DNA fragment into yeast, and then examining whether a strain carrying homologous recombination, that is, a strain having low-temperature-sensitive

fermentability can be isolated.

Suitable vectors to be used for the construction of the plasmid for the disruption of the gene complementing low-temperature-sensitivity include vectors capable of being maintained in yeast as well as vectors capable of being maintained in Escherichia coli such as pUC19, pBR322, and BluscriptII SK^+.

As the marker gene, any marker genes which can be used in yeast are usable. Examples of suitable genes are genes complementing auxotrophic mutation such as URA3, TRP1, LEU2, and HIS3, and genes relating to resistance to chemicals such as G418, hygromycin B, cerulenin, and parafluorophenylalanine [e.g. J. Ferment. Bioeng., 76, 60-63 (1993), and Enzyme and Microb. Technol., 15, 874-876 (1993)].

The gene complementing low-temperature-sensitivity on the genome of yeast can be disrupted by transforming the yeast with the plasmid for the disruption of the gene complementing low-temperature-sensitivity.

The transformation of the yeast can be carried out according to the methods generally used in genetic engineering and biological engineering such as the spheroplast method, the lithium acetate method, and the electroporation method mentioned above.

Introduction of the marker gene into the plasmid for the disruption of the gene complementing low-temperature-sensitivity enables ready isolation of a transformant by using the marker as an indicator. The transformant can also be isolated based on the exhibition of low-temperature-sensitive fermentability, which is an indication of the disruption of the gene complementing low-temperature-sensitivity on the genome of the yeast. The low-temperature-sensitivity of the strain of which the gene complementing low-temperature-sensitivity has been disrupted can be confirmed by examining the yeast for the growth or fermentability at a low temperature.

By the above-described process, yeast having low-temperature-sensitive fermentability which is characterized in that the gene complementing low-temperature-sensitivity is inactivated can be obtained. An example of such yeast is Saccharomyces cerevisiae YHK1243 (hereinafter referred to as YHK1243 strain). This strain was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken) on December 7, 1995 with accession number FERM BP-5327 under the Budapest Treaty.

The following Test Examples show that the low-temperature-sensitivity of fermentability of YHK1243 strain is lowered.

Test Example 1 Test on low-temperature-sensitivity of fermentability

One loopful of YHK1243 strain was inoculated into 5 ml of YPD medium comprising 1% yeast extract, 2% peptone and 2% glucose in a test tube, and cultured at 30°C for 16 hours. The resulting culture (1 ml) was inoculated into 50 ml of YPD medium in a 300-ml Erlenmeyer flask, and cultured at 30°C for 24 hours. After the completion of culturing, the cells were collected by centrifugation and washed twice with deionized water. The obtained wet cells (0.61 g) were suspended in 50 ml of a fermentation test medium [0.67% Yeast Nitrogen Base w/o Amino Acid (Difco Laboratories Inc.), 2% sucrose, and 1% sodium succinate (adjusted to pH 4.5 with concentrated hydrochloric acid)] in a test tube (inside diameter: 22 mm, height: 200 mm). A silicone stopper equipped with a silicone tube was put in the test tube, and culturing was carried out at 5°C for 24 hours. The gas generated during the culturing was collected in a saturated aqueous solution of sodium chloride via the silicone tube, and the volume of the gas was measured to calculate the amount of carbon dioxide gas generated per gram of yeast cells. The same procedure as above was also

carried out on YOY655 strain to calculate the amount of carbon dioxide gas generated per gram of cells.

The results are shown in Table 1.

Table 1

Strain	Amount of Carbon Dioxide Gas (ml/g of cells*)
YOY655 strain	133
YHK1243 strain	15

*: Converted as yeast cells having a dry matter content of 27%

The amount of carbon dioxide gas generated by YHK1243 strain at 5°C was approximately 1/9 of that by YOY655 strain.

Test Example 2 Test on low-temperature-sensitivity of fermentability (2)

One loopful of YHK1243 strain was inoculated into 30 ml of YPD medium in a 300-ml Erlenmeyer flask, and cultured at 30°C for 24 hours. The whole of the resulting culture was inoculated into 270 ml of a molasses medium (3% molasses, 0.193% urea, 0.046% potassium dihydrogenphosphate, and 2 drops of defoaming agent) in a 2-ℓ Erlenmeyer flask with baffles, and cultured at 30°C for 24 hours. After the completion of culturing, the cells were collected by centrifugation and washed twice with deionized water, followed by dehydration on a clay plate. The same procedure as above was also carried out on YOY655 strain to obtain cells.

The obtained cells of YHK1243 strain and YOY655 strain were respectively used for preparing dough according to the following dough composition and steps.

Dough Composition:

	(weight: g)
Hard flour	100
Sugar	5
Salt	2
Yeast cells (YHK1243 strain or YOY655 strain)	3
Water	62

Steps:

Mixing

(at 100 rpm for 2 minutes with National Complete Mixer)

↓

Dividing

(the dough is divided into five equal parts; 34.4 g each)

↓

Storage under refrigeration

(in a refrigerator at 5°C for 7 days)

↓

Thawing

(at 30°C and 85% relative humidity for 30 minutes)

↓

Measurement of the amount of carbon dioxide gas generated at 30°C in 2 hours with Fermograph (ATTO Co., Ltd.)

Each dough was stored under refrigeration, and then the amount of carbon dioxide gas generated at 30°C was measured for evaluation of the refrigeration resistance of the dough.

The results are shown in Table 2.

Table 2

Strain	Amount of Carbon Dioxide Gas (ml)	
	Before Storage under Refrigeration	After Storage under Refrigeration
YOY655 strain	124	68
YHK1243 strain	120	101

The dough containing YHK1243 strain generated a large amount of carbon dioxide gas at 30°C after the storage under refrigeration, compared with the dough containing YOY655 strain. Further, rising of the dough containing YOY655 strain was observed during the storage under refrigeration, whereas rising of the dough containing YHK1243 strain was not substantially observed.

The dough containing the yeast belonging to the genus Saccharomyces and having low-temperature-sensitive fermentability which is characterized in that the gene complementing low-temperature-sensitivity is inactivated (hereinafter referred to as the yeast of the present invention) is described below.

The dough containing the yeast of the present invention refers to the dough prepared by mixing flour or rye flour with the yeast of the present invention, salt, water, and if necessary, additional ingredients such as fats and oils, sugar, shortening, butter, skim milk, yeast food, and eggs, and kneading the mixture.

The refrigeration conditions for storing the dough containing the yeast of the present invention are as follows: at a temperature of -5 to 10°C, preferably 0 to 5°C, for 1 to 10 days, preferably 1 to 7 days.

The process for preparing the dough containing the yeast of the present invention and the process for making bread which comprises adding the yeast of the present invention to dough are described below.

Yeast cells which are suitable for use in bread-making can be obtained by culturing the yeast of the present invention in an ordinary medium containing carbon sources, nitrogen sources, inorganic substances, amino acids, vitamins, etc. at 27 to 32°C under aerobic conditions, collecting the cultured cells, and washing the cells.

Examples of the carbon sources in the medium are glucose, sucrose, starch hydrolyzate, and molasses. Particularly preferred is blackstrap molasses.

Examples of the nitrogen sources are ammonia, ammonium chloride, ammonium sulfate, ammonium carbonate, ammonium acetate, urea, yeast extract, and corn steep liquor.

Examples of the inorganic substances are magnesium phosphate and potassium phosphate. An example of the amino acids is glutamic acid, and examples of the vitamins are pantothenic acid and thiamine.

Fed-batch culture is desirable as the culturing method.

After the completion of culturing, the yeast cells of the present invention are collected by centrifugation or the like. The collected cells are added to flour or rye flour together with salt, water, and if necessary, fats and oils, sugar, shortening, butter, skim milk, yeast food, eggs, etc., followed by mixing, to prepare the dough containing the yeast of the present invention.

Bread can be made according to ordinary methods using the dough obtained as above. There are two kinds of typical methods for making one-loaf bread, buns, etc.; that is, the straight dough method and the sponge-dough method. The former is a method in which all the ingredients are mixed at a time. The latter is a method in which at first a sponge is made by kneading a part of the flour with yeast and water, and then, after fermentation, the remaining ingredients are added to the sponge.

In the straight dough method, all the ingredients are mixed and kneaded, and the kneaded mixture is fermented at 25 to 30°C. The fermented dough is subjected to the

following steps: dividing, benching, molding, proofing (35 to 42°C), and baking (200 to 240°C). In the sponge-dough method, about 70% of the whole flour to be used, yeast, and yeast food are mixed and kneaded with water. The kneaded mixture is fermented at 25 to 35°C for 3 to 5 hours, and then mixed and kneaded with the remaining ingredients such as flour, water, and salt (dough mixing). The obtained dough is subjected to the following steps: dividing, benching, molding, proofing (35 to 42°C), and baking (200 to 240°C).

Danish pastries, croissants, etc. are made, for example, in the following manner.

Flour, salt, the yeast of the present invention, sugar, shortening, eggs, skim milk, and water are mixed and kneaded to prepare dough. Then, fat such as butter or margarine is folded into the dough, and rolling and folding are repeated to make multiple layers of the dough and the fat. This step of folding the fat is called "roll-in", which can be carried out by two methods. In one method, the temperature of the dough to be kneaded is lowered to about 15°C, and the dough is kneaded until the intended number of layers are made without cooling. In the other method, which is the so-called retarding method, cooling is repeated several times using a refrigerator or a freezer in the course of the roll-in step.

The obtained dough is subjected to the following steps: rolling, dividing, molding, proofing (30 to 39°C), and baking (190 to 210°C).

The process for producing ethanol is described below which comprises culturing the yeast of the present invention in a medium, allowing ethanol to accumulate in the culture, and recovering ethanol from the culture.

The production of ethanol by using the yeast of the present invention is carried out by a conventional method for culturing yeast. The microorganism to be used in the present invention may be immobilized on a gel carrier such as agar, sodium alginate, polyacrylamide, or carageenan.

As the medium for the production of ethanol according to the present invention, either a synthetic medium or a natural medium may be used insofar as it appropriately contains carbon sources, nitrogen sources, inorganic substances, and other nutrients as required.

As the carbon sources, fermentation materials containing at least sucrose should be used. Other carbon sources which can be assimilated by the microorganism used such as sugars (e.g. glucose, fructose, galactose, and maltose) may also be used. As the fermentation materials containing sucrose, any synthetic or natural fermentation materials containing sucrose can be used; examples of suitable materials are sugarcane juice, sugar beet juice, and blackstrap molasses which is obtained after crystallization of sucrose in the process of producing sugar from such juices.

Examples of the nitrogen sources include organic or inorganic nitrogen sources such as urea, ammonia, ammonium sulfate, and ammonium nitrate, and natural nitrogen sources such as corn steep liquor, peptone, meat extract, and yeast extract.

Examples of the inorganic salts are potassium phosphate, sodium phosphate, magnesium sulfate, manganese sulfate, ferrous sulfate, potassium chloride, and sodium chloride.

As the other nutrients, vitamins such as thiamine hydrochloride, p-aminobenzoic acid, folic acid, riboflavin, and inositol, etc. can be used.

Culturing is usually carried out under aerobic conditions, for example, by shaking culture or aeration stirring culture. The culturing temperature is 25 to 50°C, preferably 30 to 43°C, and the pH is maintained at 3 to 7, preferably 4 to 6 during the culturing. Usually, the culturing is completed in 1 to 10 days.

After the completion of culturing, ethanol can be recovered from the culture by ordinary methods such as distillation.

Brief Description of the Drawings

Fig. 1 shows the restriction map of the DNA fragment containing CSF1 gene and the results of the subcloning and complementation test carried out for the determination of the functional region of CSF1 gene. Fig. 2 illustrates the steps for constructing the plasmid for the disruption of CSF1 gene.

Best Mode for Carrying Out the Invention

Example 1 Cloning of the gene complementing low-temperature-sensitivity

(1) Conferment of ura3 mutation on RZT-3 strain

RZT-3 strain, which is a yeast strain having low-temperature-sensitive fermentability, was conferred with ura3 mutation as a marker for introducing a plasmid according to the method of Boeke, et al. [Mol. Gen. Genet., 197, 345-346 (1984)]. That is, one loopful of RZT-3 strain was inoculated into YPD medium and cultured overnight at 30°C with shaking. The resulting culture (100 µl) was smeared on FOA plate [0.67% Yeast Nitrogen Base w/o Amino Acid (Difco Laboratories Inc.), 0.1% 5-fluoroorotic acid, 0.005% uracil, 2% glucose, and 2% agar], and cultured at 30°C for 3 days. From the colonies formed by the culturing was selected a strain having uracil-requirement which is complemented by transformation with plasmid YCp50 carrying URA3 as a marker, and having low-temperature-sensitive fermentability. This strain was designated Saccharomyces cerevisiae RZT-3u (hereinafter referred to as RZT-3u strain).

(2) Cloning

The chromosomal DNA of X2180-1B strain (obtained from Yeast Genetic Stock Center) was partially digested with Sau3AI, and the obtained DNA fragments were inserted into the BamHI site of plasmid YCp50 to prepare the gene library. RZT-3u strain was transformed with the gene

library, followed by selection of non-uracil-requiring transformants. The obtained transformants were cultured on YPG agar medium at 30°C to form colonies. Then, a pigment agar was layered over the medium and culturing was carried out at 5°C for 1 to 3 days. A strain showing the color change to yellow around the colony during the culturing at 5°C, that is, a strain of which the fermentation was observed at 5°C, was isolated as a strain of which the mutation exhibiting low-temperature-sensitive fermentability was complemented. From this strain was extracted recombinant plasmid pHK162.

Plasmid pHK162 was introduced into Escherichia coli JM109 strain to prepare Escherichia coli EHK162 strain. The obtained strain was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on December 7, 1995 with accession number FERM BP-5328 under the Budapest Treaty.

(3) Complementation test

Plasmid pHK162 carried an inserted Sau3AI/BamHI-BamHI fragment of about 12 Kbp. This plasmid was cleaved with various restriction enzymes and the obtained DNA fragments were separated by electrophoresis, followed by measurement of molecular weights, to prepare the restriction map as shown in Fig. 1. On the basis of this restriction map, recombinant plasmids were constructed by inserting each of the DNA fragments obtained by cleavage of the ca. 12 Kbp Sau3AI/BamHI-BamHI fragment with SphI, BamHI, MluI and ClaI into plasmid YCp50. The recombinant plasmids were used for transforming RZT-3u strain.

The obtained transformants were examined for complementation of the mutation exhibiting low-temperature-sensitive fermentability. As shown in Fig. 1, transformation of RZT-3u strain with plasmid pHK162 resulted in complementation of the mutation exhibiting low-temperature-sensitive fermentability, but transformation of

the strain with the other recombinant plasmids did not complement the mutation exhibiting low-temperature-sensitive fermentability.

The above result shows that a DNA fragment which comprises the DNA fragment of about 6.5 Kbp from BamHI (A) (the sequence at positions 1291 through 1296 in the nucleotide sequence of SEQ ID NO: 1) to SphI (B) (the sequence at positions 7675 through 7680 in the nucleotide sequence of SEQ ID NO: 1) shown in Fig. 1 and additional sequences extending upstream of the 5' end and downstream of the 3' end of the BamHI-SphI fragment is necessary for complementing the mutation exhibiting low-temperature-sensitive fermentability of RZT-3u strain.

(4) Determination of nucleotide sequence

The nucleotide sequence of the 12 Kbp DNA fragment inserted into plasmid pHK162 was determined by the dideoxy method using a DNA sequencer (Pharmacia LKB, ALF DNA Sequencer II). As a result, a gene was found which comprises the region of about 6.5 Kbp cleaved at BamHI (A) and SphI (B) shown in Fig. 1 within the open reading frame. This gene was designated CSF1 gene. As shown in the amino acid sequence of SEQ ID NO: 1, the polypeptide encoded by CSF1 gene which is presumed from the determined nucleotide sequence consists of 2958 amino acid residues (molecular weight: 338 kDa). DNA homology search with other genes revealed that the sequence of the upstream region in CSF1 gene comprising about 140 N-terminal amino acid residues in the open reading frame of CSF1 gene coincided with the sequence of the region located upstream of the sequence which was reported as the nucleotide sequence of GAA1 gene of Saccharomyces cerevisiae [Hamburger, et al.: J. Cell Biol., 129, 629-639 (1995)] (the region outside the GAA1 gene-encoding region). However, the report by Hamburger, et al. relates to GAA1 gene and contains no description about the presence of another gene (CSF1 gene) upstream from GAA1 gene. Further, in the nucleotide sequence

reported by them, one base (T) is inserted between the base at position 198 (T) and the base at position 199 (G) in the nucleotide sequence of SEQ ID NO: 1. Thus, the polypeptide encoded by CSF1 gene cannot be anticipated from the sequence reported by Hamburger, et al.

Example 2 Preparation of yeast having low-temperature-sensitive fermentability

(1) Construction of plasmid for gene disruption

About 5 μg of pHK162 plasmid DNA was dissolved in 20 μl of H buffer [50 mM Tris hydrochloride buffer (pH 7.5), 10 mM magnesium chloride, 1 mM dithiothreitol, and 100 mM sodium chloride], and 10 units of restriction enzyme BamHI was added thereto. Reaction was carried out at 30°C for 3 hours, followed by separation of the reaction product by 0.8% agarose gel electrophoresis. The segment of the gel containing the band of the DNA fragment of about 8 kb from BamHI (A) to BamHI (C) shown in Fig. 1 was cut out, and the fragment was extracted and purified by using GENECLEAN II Kit (Bio 101 Co., Ltd.). The same procedure as above was repeated except that about 5 μg of pUC19 plasmid DNA was used in place of about 5 μg of pHK162 plasmid DNA, whereby a DNA fragment of about 2.8 kb was extracted and purified. The DNA fragment of about 8 kb derived from plasmid pHK162 (1 μg) and the DNA fragment of about 2.8 kb derived from plasmid pUC19 (0.1 μg) were subjected to ligation reaction overnight at 16°C using Ligation Pack (Nippon Gene Co., Ltd.). The reaction mixture (2 μl) was used for transformation of competent high E. coli JM109 strain (Toyobo Co., Ltd.). The obtained transformant was smeared on 5-bromo-4-chloro-3-indolyl-β-D-galactoside (hereinafter referred to as X-gal) ampicillin LB agar medium and cultured at 37°C for 20 hours. The X-gal ampicillin LB agar medium was prepared by dropping 50 μl of 4% X-gal and 25 μl of isopropyl-1-thio-β-D-galactoside on LB agar medium [1% Bacto-tryptone (Difco Laboratories Inc.), 0.5% yeast

extract, 1% sodium chloride, and 1.5% agar] containing 50 μg/ml ampicillin, and spreading the drops on the medium with a spreader, followed by slight drying. After the completion of culturing, the formed white colony was isolated and cultured. A plasmid DNA was extracted and purified from the culture to obtain plasmid pHK179.

About 5 μg of pHK179 plasmid DNA was dissolved in 20 μl of H buffer, and 10 units each of restriction enzymes MluI and SpeI were added thereto. Reaction was carried out at 37°C for 3 hours. The reaction product was subjected to treatment for making blunt ends by using DNA Blunting Kit (Takara Shuzo Co., Ltd.), followed by separation by 0.8% agarose gel electrophoresis. The segment of the gel containing the band of a fragment of about 10 Kbp excluding the fragment of about 0.6 kb from MluI (the sequence at positions 4388 through 4393 in the nucleotide sequence of SEQ ID NO: 1) to SpeI (the sequence at positions 5027 through 5032 in the nucleotide sequence of SEQ ID NO: 1) shown in Fig. 1 was cut out, and the fragment was extracted and purified by using GENECLEAN II Kit. Separately, about 5 μg of YEp24 plasmid DNA, which is a vector carrying the marker gene URA3 complementing uracil-requirement mutation between the HindIII sites, was dissolved in 20 μl of M buffer [10 mM Tris hydrochloride buffer (pH 7.5), 10 mM magnesium chloride, 1 mM dithiothreitol, and 50 mM sodium chloride]. Ten units of restriction enzyme HindIII was added to the solution, and reaction was carried out at 37°C for 3 hours. The reaction product was subjected to treatment for making blunt ends by using DNA Blunting Kit (Takara Shuzo Co., Ltd.), followed by separation by 0.8% agarose gel electrophoresis. The segment of the gel containing the band of a fragment of about 1.1 kb carrying URA3 was cut out, and the fragment was extracted and purified by using GENECLEAN II Kit. The DNA fragment of about 10 kb derived from plasmid pHK179 (0.5 μg) and the DNA fragment of about 1.1 kb derived from plasmid YEp24 (0.5 μg) were subjected to ligation reaction overnight at

16°C using Ligation Pack. The reaction mixture (2 μl) was used for transformation of competent high E. coli JM109 strain. The obtained transformant was smeared on LB agar medium containing 50 μg/ml ampicillin and cultured at 37°C for 20 hours. After the completion of culturing, the formed colony was isolated and cultured. A plasmid DNA was extracted and purified from the culture to obtain plasmid pHK188 for disruption of CSF1 gene. Plasmid pHK188 was confirmed to be the desired plasmid by subjecting the plasmid to 0.8% agarose gel electrophoresis and measuring the molecular weight before and after cleavage of the plasmid with BamHI.

The outline of the steps for constructing the plasmid for the disruption of CSF1 gene is shown in Fig. 2.

(2) Disruption of CSF1 gene

Disruption of CSF1 gene carried by YOY655u strain, which is a monoploid strain of Saccharomyces cerevisiae, was carried out by using plasmid pHK188. YOY655u strain is a strain prepared by introducing uracil-requirement (ura3) mutation into YOY655 strain, which is a monoploid strain of Saccharomyces cerevisiae. The properties such as fermentability of YOY655u strain are the same as those of YOY655 strain. YOY655u strain was inoculated into 100 ml of YPD medium in an Erlenmeyer flask, and cultured with shaking at 30°C until the cell density reached 2-4 x 10^7. After the completion of culturing, the cells were collected by centrifugation (2500 rpm, 5 minutes) and then brought into contact with plasmid pHK188 by the lithium acetate method. In order to accelerate the homologous recombination of CSF1 gene with plasmid pHK188, plasmid pHK188 had been linearized by complete digestion with BamHI prior to the transformation. YOY655u strain contacted with plasmid pHK188 was inoculated on SGlu agar medium (0.67% Yeast Nitrogen Base w/o Amino Acid, 2% glucose, and 2% agar), and cultured at 30°C for 2 to 5 days. After the

completion of culturing, YHK1243 strain was obtained from one of the formed colonies as a transformant in which the uracil-requirement of YOY655u strain was complemented.

YHK1243 strain, YOY655u strain and RZT-3 strain were inoculated on YPG agar medium, and cultured at 30°C for 1 to 2 days to form colonies. Then, a pigment agar was layered over the medium, followed by culturing at 5°C for 3 days. No color change was observed around the colonies of YHK1243 strain and RZT-3 strain during the culturing, whereas the color around the colony of YOY655u strain changed to yellow on the first day of culturing.

Example 3 Process for making bread with refrigerated dough

(1) Culturing of baker's yeast

YOY655 strain and YHK1243 strain were respectively cultured in the following manner. That is, one loopful of each strain was inoculated into 30 ml of YPD medium in a 300-ml Erlenmeyer flask, and cultured at 30°C for 24 hours. The whole of the resulting culture was inoculated into 270 ml of a molasses medium (3% molasses, 0.193% urea, 0.046% potassium dihydrogenphosphate, and 2 drops of defoaming agent) in a 2-ℓ Erlenmeyer flask with baffles, and cultured at 30°C for 24 hours. After the completion of culturing, the cells were collected by centrifugation and washed twice with deionized water, followed by dehydration on a clay plate. The obtained cells were used for making bread.

(2) Preparation of bread

Bread was made according to the following dough composition and steps.

Dough Composition:

	(weight: g)
Hard flour	100
Sugar	5
Salt	2

Yeast cells	2
Water	62

Steps:

Mixing	(100 rpm, 2 minutes)
Dividing	(34.4 g)
Storage	(5°C, 7 days)
Proofing	(40°C, 90% RH, 75 minutes)
Baking	(220°C, 25 minutes)

The bread obtained using YHK1243 strain as yeast cells had a large volume compared with the bread obtained using YOY655 strain.

Example 4 Alcohol fermentation

Culturing of yeast and alcohol fermentation

YOY655 strain and YHK1243 strain were respectively cultured in the following manner. That is, one loopful of each strain was inoculated into 5 ml of YPD medium in a test tube, and cultured at 30°C for 24 hours. After the completion of culturing, 2 ml of the culture was inoculated into 20 ml of a molasses medium (25% molasses and 0.2% ammonium sulfate) in a large test tube, followed by culturing at 37°C. Samples of the culture (0.5 ml each) were taken 16 hours and 40 hours after the start of culturing and analyzed for ethanol concentration.

The results are shown in Table 3.

Table 3

Culturing Time	Ethanol production (%)	
	YOY655 strain	YHK1243 strain
16 hours	4.92*	5.37*
40 hours	10.8*	11.2*

*: The difference was significant at the 5% level of significance.

As shown in Table 3, a large amount of ethanol was produced at 37°C by the use of YHK1243 strain compared with YOY655 strain.

Industrial Applicability

The present invention provides a protein and a gene which complement the mutation exhibiting low-temperature-sensitive fermentability, refrigeration-resistant yeast which is obtained by inactivation of said gene, and processes for producing bread and ethanol using said yeast.

Sequence Listing

(1)GENERAL INFORMATION:

(i)APPLICANT: KAWASAKI, Hideki
TOKAI, Masaya
KIKUCHI, Yasuhiro
OUCHI, Kozo

(ii)TITLE OF INVENTION:NOVEL YEAST GENES

(iii)NUMBER OF SEQUENCES: 001

(iv)CORRESPONDENCE ADDRESS:
(A)ADDRESSEE:FITZPATRICK, CELLA, HARPER & SCINTO
(B)STREET: 277 Park Avenue
(C)CITY: New York
(D)STATE: New York
(E)COUNTRY: U.S.A.
(F)ZIP: 10172-0194

(v)COMPUTER READABLE FORM:
(A)MEDIUM TYPE: Diskette - 3.50 inch, 1440 Kb storage.
(B)COMPUTER: IBM PS/V
(C)OPERATING SYSTEM: MS-DOS Ver3.30
(D)SOFTWARE: PATENT AID Ver1.0

(vii)PRIOR APPLICATION DATA:
(A)APPLICATION NUMBER: JP343700/95
(B)FILING DATE: 28-DECEMBER-1995

(vii)ATTORNEY/AGENT INFORMATION:
(A)NAME: Perry, Lawrence S.
(B)REGISTRATION NUMBER: 31865

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-758-2400
(B) TELEFAX: 212-758-2982

(2) INFORMATION FOR SEQ ID NO: 1 :

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8874 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESSS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae
(B) STRAIN: X2180-1B

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1 to 8874
(C) IDENTIFICATION METHOD: E

(ix) FEATURE:
(A) NAME/KEY: cleavage-site
(B) LOCATION: 1291 to 1296
(C) IDENTIFICATION METHOD: S

(ix) FEATURE:
(A) NAME/KEY: cleavage-site
(B) LOCATION: 4388 to 4393
(C) IDENTIFICATION METHOD: S

(ix) FEATURE:
(A) NAME/KEY: cleavage-site
(B) LOCATION: 5927 to 5032

(C) IDENTIFICATION METHOD: S

(ix) FEATURE:
(A) NAME/KEY: cleavage-site
(B) LOCATION: 7675 to 7680
(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

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Asp Ile Asp Asn Val Asp Thr Leu Val Ile Glu Cys Asn Ser Lys Ser
740 745 750

ACA GTA CTT CAC TGC TAT GGG TTT GTC ATG AGG TAT TTA ACA AAC GTA 2304
Thr Val Leu His Cys Tyr Gly Phe Val Met Arg Tyr Leu Thr Asn Val
755 760 765

AAG ATG AAT TAC TTC GGT GAA TTT TTT AAT TTT GTG ACG TCA GAA GAG 2352
Lys Met Asn Tyr Phe Gly Glu Phe Phe Asn Phe Val Thr Ser Glu Glu
770 775 780

TAC ACA GGT GTC CTT GGC GCT AGG GAA GTC GGA GAT GTC ACT ACG AAA 2400
Tyr Thr Gly Val Leu Gly Ala Arg Glu Val Gly Asp Val Thr Thr Lys
785 790 795 800

AGC TCG GTG GCA GAT TTG GCA TCT ACT GTA GAT TCA GGG TAC CAA AAT 2448
Ser Ser Val Ala Asp Leu Ala Ser Thr Val Asp Ser Gly Tyr Gln Asn
805 810 815

AGC AGT CTA AAG AAC GAA TCT GAG GAT AAA GGT CCT ATG AAA AGG TCA 2496
Ser Ser Leu Lys Asn Glu Ser Glu Asp Lys Gly Pro Met Lys Arg Ser
820 825 830

GAT TTG AAA AGG ACT ACC AAC GAA ACT GAT ATT TGG TTC ACA TTT TCG 2544
Asp Leu Lys Arg Thr Thr Asn Glu Thr Asp Ile Trp Phe Thr Phe Ser
835 840 845

GTT TGG GAT GGT GCT CTG ATA TTA CCA GAA ACG ATT TAC AGT TTT GAT 2592
Val Trp Asp Gly Ala Leu Ile Leu Pro Glu Thr Ile Tyr Ser Phe Asp
850 855 860

CCA TGC ATT GCA CTA CAT TTT GCC GAA CTT GTA GTG GAT TTC AGA AGT 2640
Pro Cys Ile Ala Leu His Phe Ala Glu Leu Val Val Asp Phe Arg Ser
865 870 875 880

TGT AAT TAT TAT ATG GAC ATA ATG GCG GTT CTC AAC GGG ACT TCA ATA 2688
Cys Asn Tyr Tyr Met Asp Ile Met Ala Val Leu Asn Gly Thr Ser Ile
885 890 895

AAG CGG CAC GTT TCA AAA CAA ATA AAT GAA GTA TTT GAT TTT ATA CGT 2736
Lys Arg His Val Ser Lys Gln Ile Asn Glu Val Phe Asp Phe Ile Arg
900 905 910

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CGT AAT AAC GGA GCT GAT GAG CAA GAG CAC GGA TTG CTT TCG GAC CTC   2784
Arg Asn Asn Gly Ala Asp Glu Gln Glu His Gly Leu Leu Ser Asp Leu
        915                 920                 925
ACC ATT CAT GGA CAT AGA ATG TAT GGA TTA CCA CCC ACA GAA CCT ACC   2832
Thr Ile His Gly His Arg Met Tyr Gly Leu Pro Pro Thr Glu Pro Thr
    930                 935                 940
TAC TTT TGT CAA TGG GAT ATC AAT CTC GGA GAT TTA TGC ATT GAT TCA   2880
Tyr Phe Cys Gln Trp Asp Ile Asn Leu Gly Asp Leu Cys Ile Asp Ser
945                 950                 955                 960
GAT ATT GAA TTT ATA AAG GGA TTC TTT AAT TCC TTT TAT AAG ATA GGT   2928
Asp Ile Glu Phe Ile Lys Gly Phe Phe Asn Ser Phe Tyr Lys Ile Gly
                965                 970                 975
TTT GGC TAC AAT GAC TTG GAA AAT ATA TTA TTA TAT GAC ACT GAG ACC   2976
Phe Gly Tyr Asn Asp Leu Glu Asn Ile Leu Leu Tyr Asp Thr Glu Thr
            980                 985                 990
ATT AAT GAT ATG ACC TCG CTA ACC GTG CAC GTT GAA AAA ATA AGA ATA   3024
Ile Asn Asp Met Thr Ser Leu Thr Val His Val Glu Lys Ile Arg Ile
        995                 1000                1005
GGC CTT AAA GAT CCG GTG ATG AAA TCT CAA TCA GTT ATT AGT GCT GAA   3072
Gly Leu Lys Asp Pro Val Met Lys Ser Gln Ser Val Ile Ser Ala Glu
    1010                1015                1020
TCG ATA TTG TTT ACT TTG ATC GAC TTT GAA AAC GAA AAA TAT TCA CAA   3120
Ser Ile Leu Phe Thr Leu Ile Asp Phe Glu Asn Glu Lys Tyr Ser Gln
1025                1030                1035                1040
AGA ATA GAC GTG AAA ATT CCA AAA TTG ACA ATT TCG TTA AAT TGC GTG   3168
Arg Ile Asp Val Lys Ile Pro Lys Leu Thr Ile Ser Leu Asn Cys Val
                1045                1050                1055
ATG GGC GAT GGC GTA GAC ACA TCA TTT CTC AAA TTC GAA ACA AAA TTA   3216
Met Gly Asp Gly Val Asp Thr Ser Phe Leu Lys Phe Glu Thr Lys Leu
            1060                1065                1070
AGA TTT ACA AAC TTT GAG CAA TAC AAG GAT ATC GAT AAA AAA AGA TCA   3264
Arg Phe Thr Asn Phe Glu Gln Tyr Lys Asp Ile Asp Lys Lys Arg Ser
        1075                1080                1085
GAA CAA CGC AGA TAT ATA ACA ATA CAC GAT TCA CCC TAT CAT AGG TGT   3312
Glu Gln Arg Arg Tyr Ile Thr Ile His Asp Ser Pro Tyr His Arg Cys
    1090                1095                1100
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CCT TTT CTT CTT CCG CTG TTC TAT CAG GAT TCG GAT ACA TAC CAA AAC 3360
Pro Phe Leu Leu Pro Leu Phe Tyr Gln Asp Ser Asp Thr Tyr Gln Asn
1105 1110 1115 1120

CTG TAC GGG GCT ATA GCA CCA TCT TCG TCT ATC CCA ACT TTA CCT CTT 3408
Leu Tyr Gly Ala Ile Ala Pro Ser Ser Ser Ile Pro Thr Leu Pro Leu
1125 1130 1135

CCC ACT TTG CCT GAT ACT ATA GAT TAT ATC ATT GAA GAT ATT GTG GGC 3456
Pro Thr Leu Pro Asp Thr Ile Asp Tyr Ile Ile Glu Asp Ile Val Gly
1140 1145 1150

GAG TAT GCT ACC CTT CTG GAG ACC ACA AAT CCA TTC AAG AAC ATA TTC 3504
Glu Tyr Ala Thr Leu Leu Glu Thr Thr Asn Pro Phe Lys Asn Ile Phe
1155 1160 1165

GCA GAA ACT CCA TCA ACT ATG GAG CCT TCA AGA GCC AGC TTC AGT GAA 3552
Ala Glu Thr Pro Ser Thr Met Glu Pro Ser Arg Ala Ser Phe Ser Glu
1170 1175 1180

GAT GAT AAT GAC GAA GAA GCG GAC CCT TCA AGC TTC AAA CCT GTC GCT 3600
Asp Asp Asn Asp Glu Glu Ala Asp Pro Ser Ser Phe Lys Pro Val Ala
1185 1190 1195 1200

TTT ACA GAA GAC AGA AAC CAC GAA AGG GAT AAC TAT GTT GTT GAT GTT 3648
Phe Thr Glu Asp Arg Asn His Glu Arg Asp Asn Tyr Val Val Asp Val
1205 1210 1215

TCA TAT ATT CTG TTG GAT GTC GAC CCG TTG CTT TTT ATT TTC GCT AAG 3696
Ser Tyr Ile Leu Leu Asp Val Asp Pro Leu Leu Phe Ile Phe Ala Lys
1220 1225 1230

AGT TTA TTA GAA CAG CTT TAC TCT GAA AAC ATG GTA CAA GTC TTA GAC 3744
Ser Leu Leu Glu Gln Leu Tyr Ser Glu Asn Met Val Gln Val Leu Asp
1235 1240 1245

GAT ATT GAA ATT GGG ATT GTG AAA CGA TTA AGC AAC CTT CAA GAA GGG 3792
Asp Ile Glu Ile Gly Ile Val Lys Arg Leu Ser Asn Leu Gln Glu Gly
1250 1255 1260

ATC ACT TCT ATT TCA AAC ATT GAT ATC CAT ATT GCT TAT CTA AAT TTA 3840
Ile Thr Ser Ile Ser Asn Ile Asp Ile His Ile Ala Tyr Leu Asn Leu
1265 1270 1275 1280

ATC TGG CAA GAG ACA GGT GAG GAA GGT TTT GAG CTC TAT TTA GAT CGT 3888
Ile Trp Gln Glu Thr Gly Glu Glu Gly Phe Glu Leu Tyr Leu Asp Arg
1285 1290 1295

ATT GAT TAT CAA ATG AGT GAA AAG TCT CTA GAG AAG AAC CGA ACA AAT 3936
Ile Asp Tyr Gln Met Ser Glu Lys Ser Leu Glu Lys Asn Arg Thr Asn
1300 1305 1310

AAA TTA TTA GAA GTA GCA GCT TTA GCA AAG GTA AAA ACT GTC AGA GTG 3984
Lys Leu Leu Glu Val Ala Ala Leu Ala Lys Val Lys Thr Val Arg Val
1315 1320 1325

ACT GTT AAC CAG AAG AAA AAT CCA GAC TTG TCT GAA GAT CGT CCC CCT 4032
Thr Val Asn Gln Lys Lys Asn Pro Asp Leu Ser Glu Asp Arg Pro Pro
1330 1335 1340

GCA CTG TCG CTA GGG ATT GAG GGT TTC GAA GTA TGG TCT TCT ACA GAA 4080
Ala Leu Ser Leu Gly Ile Glu Gly Phe Glu Val Trp Ser Ser Thr Glu
1345 1350 1355 1360

GAT AGA CAA GTT AAC TCA TTA AAC TTA ACG TCA TCA GAT ATT ACC ATA 4128
Asp Arg Gln Val Asn Ser Leu Asn Leu Thr Ser Ser Asp Ile Thr Ile
1365 1370 1375

GAC GAA TCT CAA ATG GAA TGG CTG TTT GAG TAC TGT AGT GAC CAG GGA 4176
Asp Glu Ser Gln Met Glu Trp Leu Phe Glu Tyr Cys Ser Asp Gln Gly
1380 1385 1390

AAT CTT ATT CAA GAG GTT TGC ACT TCT TTT AAT TCT ATT CAG AAC ACC 4224
Asn Leu Ile Gln Glu Val Cys Thr Ser Phe Asn Ser Ile Gln Asn Thr
1395 1400 1405

AGA AGT AAT TCA AAG ACA GAA CTC ATT TCA AAG CTC ACA GCC GCA AGC 4272
Arg Ser Asn Ser Lys Thr Glu Leu Ile Ser Lys Leu Thr Ala Ala Ser
1410 1415 1420

GAA TAT TAT CAA ATT AGT CAT GAT CCT TAC GTC ATA ACA AAA CCT GCT 4320
Glu Tyr Tyr Gln Ile Ser His Asp Pro Tyr Val Ile Thr Lys Pro Ala
1425 1430 1435 1440

TTT ATT ATG AGA CTT TCC AAA GGG CAT GTG CGT GAG AAT CGT AGT TGG 4368
Phe Ile Met Arg Leu Ser Lys Gly His Val Arg Glu Asn Arg Ser Trp
1445 1450 1455

AAA ATT ATT ACG CGT CTG AGA CAC ATT TTA ACG TAC CTT CCT GAT GAT 4416
Lys Ile Ile Thr Arg Leu Arg His Ile Leu Thr Tyr Leu Pro Asp Asp
1460 1465 1470

TGG CAA AGC AAC ATC GAC GAA GTG CTA AAA GAA AAG AAA TAT ACC TCT 4464
Trp Gln Ser Asn Ile Asp Glu Val Leu Lys Glu Lys Lys Tyr Thr Ser
1475 1480 1485

GCT AAA GAT GCA AAA AAT ATC TTC ATG TCT GTG TTT TCG ACT TGG AGA 4512
Ala Lys Asp Ala Lys Asn Ile Phe Met Ser Val Phe Ser Thr Trp Arg
1490 1495 1500
AAT TGG GAG TTC TCA GAT GTT GCA AGG TCG TAT ATA TAC GGC AAA TTA 4560
Asn Trp Glu Phe Ser Asp Val Ala Arg Ser Tyr Ile Tyr Gly Lys Leu
1505 1510 1515 1520
TTC ACG GCA GAA AAT GAG AAA CAT AAA CAA AAT TTG ATT AAA AAA TTG 4608
Phe Thr Ala Glu Asn Glu Lys His Lys Gln Asn Leu Ile Lys Lys Leu
1525 1530 1535
TTG AAG TGT ACC ATG GGA TCA TTT TAC CTT ACT GTT TAT GGT GAG GGA 4656
Leu Lys Cys Thr Met Gly Ser Phe Tyr Leu Thr Val Tyr Gly Glu Gly
1540 1545 1550
TAT GAG GTT GAG CAT AAT TTT GTT GTT GCG GAT GCC AAT CTG GTA GTG 4704
Tyr Glu Val Glu His Asn Phe Val Val Ala Asp Ala Asn Leu Val Val
1555 1560 1565
GAT TTG ACG CCT CCG GTG ACA AGC TTA CCT TCA AAT CGA GAA GAA ACT 4752
Asp Leu Thr Pro Pro Val Thr Ser Leu Pro Ser Asn Arg Glu Glu Thr
1570 1575 1580
ATT GAA ATT ACG GGA AGA GTA GGC TCA GTA AAA GGA AAA TTC AGT GAT 4800
Ile Glu Ile Thr Gly Arg Val Gly Ser Val Lys Gly Lys Phe Ser Asp
1585 1590 1595 1600
AGG TTA CTT AAA TTG CAA GAT CTT ATT CCA CTC ATT GCA GCA GTG GGC 4848
Arg Leu Leu Lys Leu Gln Asp Leu Ile Pro Leu Ile Ala Ala Val Gly
1605 1610 1615
GAA GAT GAC AAA AGT GAT CCA AAA AAG GAG TTA TCA AAG CAA TTC AAA 4896
Glu Asp Asp Lys Ser Asp Pro Lys Lys Glu Leu Ser Lys Gln Phe Lys
1620 1625 1630
ATG AAC ACC GTT TTA TTA GTG GAT AAA AGT GAA CTG CAA CTG GTC ATG 4944
Met Asn Thr Val Leu Leu Val Asp Lys Ser Glu Leu Gln Leu Val Met
1635 1640 1645
GAC CAA ACG AAG CTG ATG AGT AGA ACA GTT GGG GGT AGA GTT AGT TTA 4992
Asp Gln Thr Lys Leu Met Ser Arg Thr Val Gly Gly Arg Val Ser Leu
1650 1655 1660
CTA TGG GAA AAT CTA AAA GAT TCA ACT AGT CAA GCG GGT TCA TTG GTT 5040
Leu Trp Glu Asn Leu Lys Asp Ser Thr Ser Gln Ala Gly Ser Leu Val
1665 1670 1675 1680

ATA TTT TCC CAG AAA TCG GAA GTG TGG TTA AAA CAC ACA TCT GTC ATT 5088
Ile Phe Ser Gln Lys Ser Glu Val Trp Leu Lys His Thr Ser Val Ile
1685 1690 1695

TTG GGA GAA GCT CAA CTG CGC GAC TTT TCA GTT TTA GCG ACT ACT GAG 5136
Leu Gly Glu Ala Gln Leu Arg Asp Phe Ser Val Leu Ala Thr Thr Glu
1700 1705 1710

GCA TGG TCA CAC AAG CCT ACG ATT CTG ATA AAC AAC CAG TGC GCA GAT 5184
Ala Trp Ser His Lys Pro Thr Ile Leu Ile Asn Asn Gln Cys Ala Asp
1715 1720 1725

CTT CAT TTT AGA GCA ATG AGT TCA ACT GAG CAA TTA GTA ACC GCT ATT 5232
Leu His Phe Arg Ala Met Ser Ser Thr Glu Gln Leu Val Thr Ala Ile
1730 1735 1740

ACT GAA ATT AGG GAA AGT CTG ATG ATG ATT AAA GAG CGC ATA AAG TTT 5280
Thr Glu Ile Arg Glu Ser Leu Met Met Ile Lys Glu Arg Ile Lys Phe
1745 1750 1755 1760

AAA CCT AAA TCA AAG AAA AAG TCC CAA TTT GTC GAC CAG AAA ATT AAT 5328
Lys Pro Lys Ser Lys Lys Lys Ser Gln Phe Val Asp Gln Lys Ile Asn
1765 1770 1775

ACA GTC TTG TCA TGT TAT TTT TCA AAC GTT AGT TCT GAA GTT ATG CCG 5376
Thr Val Leu Ser Cys Tyr Phe Ser Asn Val Ser Ser Glu Val Met Pro
1780 1785 1790

CTC TCG CCA TTT TAT ATT CGT CAC GAA GCC AAG CAG CTT GAT ATA TAT 5424
Leu Ser Pro Phe Tyr Ile Arg His Glu Ala Lys Gln Leu Asp Ile Tyr
1795 1800 1805

TTT AAC AAA TTC GGT TCA AAT GAG ATT TTG TTA AGC ATA TGG GAT ACT 5472
Phe Asn Lys Phe Gly Ser Asn Glu Ile Leu Leu Ser Ile Trp Asp Thr
1810 1815 1820

GAT TTT TTC ATG ACA TCG CAC CAG ACA AAG GAG CAA TAC CTA AGG TTT 5520
Asp Phe Phe Met Thr Ser His Gln Thr Lys Glu Gln Tyr Leu Arg Phe
1825 1830 1835 1840

TCA TTT GGC GAT ATT GAA ATT AAA GGA GGA ATT TCT AGA GAA GGC TAT 5568
Ser Phe Gly Asp Ile Glu Ile Lys Gly Gly Ile Ser Arg Glu Gly Tyr
1845 1850 1855

TCG TTG ATA AAC GTT GAC ATC TCA ATA TCT ATG ATT AAG TTA ACC TTT 5616
Ser Leu Ile Asn Val Asp Ile Ser Ile Ser Met Ile Lys Leu Thr Phe
1860 1865 1870

TCG GAG CCG CGC CGT ATT GTA AAC AGT TTT TTA CAA GAT GAA AAG CTT 5664

Ser Glu Pro Arg Arg Ile Val Asn Ser Phe Leu Gln Asp Glu Lys Leu

1875 1880 1885

GCT TCT CAG GGT ATC AAT CTG TTA TAT TCC CTG AAG CCT TTA TTC TTT 5712

Ala Ser Gln Gly Ile Asn Leu Leu Tyr Ser Leu Lys Pro Leu Phe Phe

1890 1895 1900

AGT TCA AAT CTA CCA AAA AAA GAG AAG CAG GCA CCC TCG ATA ATG ATA 5760

Ser Ser Asn Leu Pro Lys Lys Glu Lys Gln Ala Pro Ser Ile Met Ile

1905 1910 1915 1920

AAT TGG ACA TTA GAT ACT AGC ATT ACT TAT TTT GGT GTT CTT GTG CCA 5808

Asn Trp Thr Leu Asp Thr Ser Ile Thr Tyr Phe Gly Val Leu Val Pro

1925 1930 1935

GTG GCT TCC ACG TAT TTC GTG TTT GAA TTA CAT ATG CTG CTA CTT TCT 5856

Val Ala Ser Thr Tyr Phe Val Phe Glu Leu His Met Leu Leu Leu Ser

1940 1945 1950

CTG ACC AAT ACG AAT AAC GGT ATG TTA CCA GAA GAA ACC AAG GTG ACG 5904

Leu Thr Asn Thr Asn Asn Gly Met Leu Pro Glu Glu Thr Lys Val Thr

1955 1960 1965

GGA CAG TTT TCC ATC GAA AAC ATC CTA TTT CTA ATA AAG GAG CGG TCA 5952

Gly Gln Phe Ser Ile Glu Asn Ile Leu Phe Leu Ile Lys Glu Arg Ser

1970 1975 1980

CTA CCC ATT GGT CTT TCC AAA TTA CTC GAC TTT TCC ATA AAA GTA TCA 6000

Leu Pro Ile Gly Leu Ser Lys Leu Leu Asp Phe Ser Ile Lys Val Ser

1985 1990 1995 2000

ACC CTA CAA AGA ACG GTT GAT ACG GAG CAG TCA TTC CAA GTG GAA AGT 6048

Thr Leu Gln Arg Thr Val Asp Thr Glu Gln Ser Phe Gln Val Glu Ser

2005 2010 2015

TCT CAT TTC AGG GTC TGC TTA TCT CCT GAT TCT CTA TTA AGA TTA ATG 6096

Ser His Phe Arg Val Cys Leu Ser Pro Asp Ser Leu Leu Arg Leu Met

2020 2025 2030

TGG GGC GCG CAT AAA TTG CTA GAC TTG AGC CAT TAC TAT TCA AGA CGC 6144

Trp Gly Ala His Lys Leu Leu Asp Leu Ser His Tyr Tyr Ser Arg Arg

2035 2040 2045

CAT GCC CCT AAT ATT TGG AAC ACT AAG ATG TTC ACC GGT AAA AGT GAT 6192

His Ala Pro Asn Ile Trp Asn Thr Lys Met Phe Thr Gly Lys Ser Asp

2050 2055 2060

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AAG TCA AAA GAA ATG CCC ATA AAT TTC CGT TCA ATA CAC ATC CTG TCC   6240
Lys Ser Lys Glu Met Pro Ile Asn Phe Arg Ser Ile His Ile Leu Ser
2065                2070                2075                2080
TAT AAA TTT TGT ATT GGG TGG ATA TTC CAG TAT GGA GCA GGC TCC AAT   6288
Tyr Lys Phe Cys Ile Gly Trp Ile Phe Gln Tyr Gly Ala Gly Ser Asn
                    2085                2090                2095
CCT GGG TTA ATG TTA GGT TAT AAC AGA TTG TTT TCA GCA TAT GAA AAG   6336
Pro Gly Leu Met Leu Gly Tyr Asn Arg Leu Phe Ser Ala Tyr Glu Lys
            2100                2105                2110
GAT TTT GGG AAA TTC ACA GTT GTG GAC GCT TTT TTC TCT GTT GCG AAT   6384
Asp Phe Gly Lys Phe Thr Val Val Asp Ala Phe Phe Ser Val Ala Asn
        2115                2120                2125
GGT AAT ACC TCA AGC ACT TTT TTC TCT GAA GGA AAC GAG AAA GAC AAA   6432
Gly Asn Thr Ser Ser Thr Phe Phe Ser Glu Gly Asn Glu Lys Asp Lys
    2130                2135                2140
TAT AAT AGA AGT TTC TTG CCA AAC ATG CAA ATA TCC TAC TGG TTC AAA   6480
Tyr Asn Arg Ser Phe Leu Pro Asn Met Gln Ile Ser Tyr Trp Phe Lys
2145                2150                2155                2160
AGA TGT GGT GAG TTG AAA GAT TGG TTT TTT AGA TTT CAT GGT GAA GCA   6528
Arg Cys Gly Glu Leu Lys Asp Trp Phe Phe Arg Phe His Gly Glu Ala
                    2165                2170                2175
CTG GAT GTA AAC TTT GTC CCG TCA TTC ATG GAT GTC ATT GAG TCT ACT   6576
Leu Asp Val Asn Phe Val Pro Ser Phe Met Asp Val Ile Glu Ser Thr
            2180                2185                2190
TTA CAA TCC ATG CGA GCA TTT CAA GAG CTG AAA AAG AAC ATT CTG GAT   6624
Leu Gln Ser Met Arg Ala Phe Gln Glu Leu Lys Lys Asn Ile Leu Asp
        2195                2200                2205
GTG TCC GAG AGT TTG CGT GCG GAA AAT GAT AAT TCT TAT GCT AGT ACC   6672
Val Ser Glu Ser Leu Arg Ala Glu Asn Asp Asn Ser Tyr Ala Ser Thr
    2210                2215                2220
AGT GTC GAA AGT GCT TCG AGT AGT TTG GCT CCC TTT CTC GAT AAC ATT   6720
Ser Val Glu Ser Ala Ser Ser Ser Leu Ala Pro Phe Leu Asp Asn Ile
2225                2230                2235                2240
AGA TCT GTT AAC TCA AAT TTC AAG TAT GAC GGT GGT GTA TTT AGG GTT   6768
Arg Ser Val Asn Ser Asn Phe Lys Tyr Asp Gly Gly Val Phe Arg Val
                    2245                2250                2255
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TAC ACG TAC GAA GAT ATT GAA ACC AAG AGT GAG CCA TCT TTT GAA ATA 6816
Tyr Thr Tyr Glu Asp Ile Glu Thr Lys Ser Glu Pro Ser Phe Glu Ile
2260 2265 2270

AAA AGT CCA GTA GTC ACT ATA AAC TGT ACA TAT AAA CAT GAT GAA GAT 6864
Lys Ser Pro Val Val Thr Ile Asn Cys Thr Tyr Lys His Asp Glu Asp
2275 2280 2285

AAA GTT AAG CCA CAT AAA TTC AGA ACA TTA ATC ACT GTC GAC CCA ACG 6912
Lys Val Lys Pro His Lys Phe Arg Thr Leu Ile Thr Val Asp Pro Thr
2290 2295 2300

CAT AAT ACT TTG TAT GCG GGA TGT GCT CCT TTA TTA ATG GAA TTT TCT 6960
His Asn Thr Leu Tyr Ala Gly Cys Ala Pro Leu Leu Met Glu Phe Ser
2305 2310 2315 2320

GAA AGT CTG CAA AAG ATG ATA AAG AAA CAT AGC ACC GAC GAA AAA CCA 7008
Glu Ser Leu Gln Lys Met Ile Lys Lys His Ser Thr Asp Glu Lys Pro
2325 2330 2335

AAC TTT ACA AAA CCT TCT TCA CAG AAT GTT GAT TAT AAG CGA CTT TTG 7056
Asn Phe Thr Lys Pro Ser Ser Gln Asn Val Asp Tyr Lys Arg Leu Leu
2340 2345 2350

GAT CAA TTT GAT GTG GCT GTA AAA CTA ACA TCA GCC AAG CAA CAG CTA 7104
Asp Gln Phe Asp Val Ala Val Lys Leu Thr Ser Ala Lys Gln Gln Leu
2355 2360 2365

AGT TTG AGC TGT GAA CCA AAA GCT AAG GTT CAG GCA GAT GTT GGA TTT 7152
Ser Leu Ser Cys Glu Pro Lys Ala Lys Val Gln Ala Asp Val Gly Phe
2370 2375 2380

GAA TCG TTT TTG TTC AGT ATG GCT ACC AAT GAG TTC GAC TCT GAA CAG 7200
Glu Ser Phe Leu Phe Ser Met Ala Thr Asn Glu Phe Asp Ser Glu Gln
2385 2390 2395 2400

CCT TTG GAG TTT TCT TTA ACT CTA GAA CAC ACA AAA GCG TCC ATT AAG 7248
Pro Leu Glu Phe Ser Leu Thr Leu Glu His Thr Lys Ala Ser Ile Lys
2405 2410 2415

CAC ATA TTT TCA AGA GAA GTA AGT ACG TCC TTT GAA GTT GGT TTC ATG 7296
His Ile Phe Ser Arg Glu Val Ser Thr Ser Phe Glu Val Gly Phe Met
2420 2425 2430

GAC TTG ACG CTT TTA TTT ACA CAT CCT GAT GTA ATC AGT ATG TAT GGA 7344
Asp Leu Thr Leu Leu Phe Thr His Pro Asp Val Ile Ser Met Tyr Gly
2435 2440 2445

ACG GGG TTG GTT TCT GAT CTA AGC GTC TTC TTC AAT GTA AAG CAG CTC 7392
Thr Gly Leu Val Ser Asp Leu Ser Val Phe Phe Asn Val Lys Gln Leu
2450 2455 2460
CAG AAC CTG TAT TTA TTC TTG GAC ATA TGG AGG TTC AGT AGC ATT TTA 7440
Gln Asn Leu Tyr Leu Phe Leu Asp Ile Trp Arg Phe Ser Ser Ile Leu
2465 2470 2475 2480
CAC ACA CGG CCA GTG CAA AGA ACT GTT AAT AAG GAA ATT GAA ATG AGT 7488
His Thr Arg Pro Val Gln Arg Thr Val Asn Lys Glu Ile Glu Met Ser
2485 2490 2495
TCA TTA ACA TCA ACC AAC TAT GCC GAT GCA GGT ACG GAA ATA CCC TGG 7536
Ser Leu Thr Ser Thr Asn Tyr Ala Asp Ala Gly Thr Glu Ile Pro Trp
2500 2505 2510
TGC TTT ACA TTA ATT TTT ACA AAT GTT AGC GGA GAC GTT GAT TTG GGT 7584
Cys Phe Thr Leu Ile Phe Thr Asn Val Ser Gly Asp Val Asp Leu Gly
2515 2520 2525
CCT TCT CTC GGT ATG ATT TCA TTA AGG ACA CAA AGA ACA TGG CTG GCC 7632
Pro Ser Leu Gly Met Ile Ser Leu Arg Thr Gln Arg Thr Trp Leu Ala
2530 2535 2540
ACA GAT CAT TAT AAC GAG AAG CGG CAG TTA CTG CAT GCT TTC ACT GAC 7680
Thr Asp His Tyr Asn Glu Lys Arg Gln Leu Leu His Ala Phe Thr Asp
2545 2550 2555 2560
GGT ATT AGC TTG ACA TCA GAA GGT AGA CTG AGT GGT TTA TTT GAA GTT 7728
Gly Ile Ser Leu Thr Ser Glu Gly Arg Leu Ser Gly Leu Phe Glu Val
2565 2570 2575
GCG AAT GCA AGT TGG TTA TCA GAA GTA AAA TGG CCA CCT GAA AAA AGC 7776
Ala Asn Ala Ser Trp Leu Ser Glu Val Lys Trp Pro Pro Glu Lys Ser
2580 2585 2590
AAA AAT ACT CAT CCA TTA GTT TCC ACC TCC CTG AAT ATT GAT GAT ATA 7824
Lys Asn Thr His Pro Leu Val Ser Thr Ser Leu Asn Ile Asp Asp Ile
2595 2600 2605
GCG GTA AAG GCT GCT TTT GAT TAT CAT ATG TTC TTA ATC GGC ACT ATA 7872
Ala Val Lys Ala Ala Phe Asp Tyr His Met Phe Leu Ile Gly Thr Ile
2610 2615 2620
AGT AAC ATA CAC TTC CAT CTT CAT AAT GAA AAG GAT GCC AAG GGG GTT 7920
Ser Asn Ile His Phe His Leu His Asn Glu Lys Asp Ala Lys Gly Val
2625 2630 2635 2640

CTA CCT GAT TTG CTG CAG GTC TCT TTT TCA TCA GAT GAA ATT ATC CTC 7968
Leu Pro Asp Leu Leu Gln Val Ser Phe Ser Ser Asp Glu Ile Ile Leu
2645 2650 2655
AGC TCT ACT GCA TTA GTT GTA GCA AAT ATA CTG GAT ATC TAC AAC ACC 8016
Ser Ser Thr Ala Leu Val Val Ala Asn Ile Leu Asp Ile Tyr Asn Thr
2660 2665 2670
ATT GTA CGT ATG AGG CAG GAT AAT AAA ATA TCG TAT ATG GAG ACG TTG 8064
Ile Val Arg Met Arg Gln Asp Asn Lys Ile Ser Tyr Met Glu Thr Leu
2675 2680 2685
AGA GAT TCC AAT CCT GGT GAA TCT AGG CAA CCA ATA TTA TAC AAA GAC 8112
Arg Asp Ser Asn Pro Gly Glu Ser Arg Gln Pro Ile Leu Tyr Lys Asp
2690 2695 2700
ATT TTA AGA TCG CTG AAA TTA CTC AGA ACT GAT CTC TCG GTG AAT ATC 8160
Ile Leu Arg Ser Leu Lys Leu Leu Arg Thr Asp Leu Ser Val Asn Ile
2705 2710 2715 2720
TCC TCT TCA AAG GTC CAG ATT TCG CCA ATA TCT TTA TTC GAT GTG GAA 8208
Ser Ser Ser Lys Val Gln Ile Ser Pro Ile Ser Leu Phe Asp Val Glu
2725 2730 2735
GTG TTA GTA ATA AGA ATT GAC AAA GTC TCT ATA CGT TCC GAA ACA CAT 8256
Val Leu Val Ile Arg Ile Asp Lys Val Ser Ile Arg Ser Glu Thr His
2740 2745 2750
TCG GGG AAA AAA TTA AAG ACA GAT TTG CAA CTA CAA GTT TTA GAT GTT 8304
Ser Gly Lys Lys Leu Lys Thr Asp Leu Gln Leu Gln Val Leu Asp Val
2755 2760 2765
TCT GCA GCG CTT TCT ACT TCC AAA GAA GAA TTA GAT GAG GAA GTT GGA 8352
Ser Ala Ala Leu Ser Thr Ser Lys Glu Glu Leu Asp Glu Glu Val Gly
2770 2775 2780
GCT TCC ATT GCT ATT GAT GAT TAC ATG CAT TAT GCT TCC AAG ATT GTC 8400
Ala Ser Ile Ala Ile Asp Asp Tyr Met His Tyr Ala Ser Lys Ile Val
2785 2790 2795 2800
GGT GGT ACT ATC ATT GAT ATT CCA AAA CTT GCT GTT CAT ATG ACA ACT 8448
Gly Gly Thr Ile Ile Asp Ile Pro Lys Leu Ala Val His Met Thr Thr
2805 2810 2815
TTA CAA GAA GAA AAG ACA AAT AAT TTA GAA TAT CTA TTT GCT TGC TCT 8496
Leu Gln Glu Glu Lys Thr Asn Asn Leu Glu Tyr Leu Phe Ala Cys Ser
2820 2825 2830

TTT TCA GAC AAA ATA TCT GTA AGG TGG AAT CTA GGG CCT GTA GAC TTC 8544
Phe Ser Asp Lys Ile Ser Val Arg Trp Asn Leu Gly Pro Val Asp Phe
2835 2840 2845
ATA AAG GAA ATG TGG ACT ACA CAT GTC AAA GCA CTG GCA GTT CGT CGA 8592
Ile Lys Glu Met Trp Thr Thr His Val Lys Ala Leu Ala Val Arg Arg
2850 2855 2860
TCC CAG GTA GCA AAT ATT TCC TTT GGA CAA ACT GAG GAA GAA CTT GAA 8640
Ser Gln Val Ala Asn Ile Ser Phe Gly Gln Thr Glu Glu Glu Leu Glu
2865 2870 2875 2880
GAA TCA ATT AAA AAG GAA GAA GCC GCT TCA AAG TTT AAT TAT ATT GCA 8688
Glu Ser Ile Lys Lys Glu Glu Ala Ala Ser Lys Phe Asn Tyr Ile Ala
2885 2890 2895
CTA GAA GAA CCG CAG ATC GAA GTG CCT CAG ATA AGA GAT CTG GGA GAC 8736
Leu Glu Glu Pro Gln Ile Glu Val Pro Gln Ile Arg Asp Leu Gly Asp
2900 2905 2910
GCC ACT CCA CCT ATG GAA TGG TTT GGT GTC AAT AGA AAA AAA TTT CCG 8784
Ala Thr Pro Pro Met Glu Trp Phe Gly Val Asn Arg Lys Lys Phe Pro
2915 2920 2925
AAA TTC ACT CAC CAA ACC GCA GTT ATC CCC GTC CAA AAG CTT GTT TAT 8832
Lys Phe Thr His Gln Thr Ala Val Ile Pro Val Gln Lys Leu Val Tyr
2930 2935 2940
CTT GCA GAA AAG CAG TAT GTC AAG ATA CTA GAT GAT ACG CAT 8874
Leu Ala Glu Lys Gln Tyr Val Lys Ile Leu Asp Asp Thr His
2945 2950 2955

CLAIMS

1. A gene which encodes a protein having the amino acid sequence represented by SEQ ID NO: 1, or encodes a protein being capable of complementing the mutation exhibiting low-temperature-sensitive fermentability and having an amino acid sequence wherein one or more amino acid residues are added, deleted or substituted in the amino acid sequence represented by SEQ ID NO: 1.

2. A gene which comprises DNA having the nucleotide sequence represented by SEQ ID NO: 1, or comprises DNA being capable of complementing the mutation exhibiting low-temperature-sensitive fermentability and having a nucleotide sequence wherein one or more DNAs are added, deleted or substituted in the nucleotide sequence represented by SEQ ID NO: 1.

3. A protein having the amino acid sequence represented by SEQ ID NO: 1, or a protein being capable of complementing the mutation exhibiting low-temperature-sensitive fermentability and having an amino acid sequence wherein one or more amino acid residues are added, deleted or substituted in the amino acid sequence represented by SEQ ID NO: 1.

4. Yeast belonging to the genus Saccharomyces and having low-temperature-sensitive fermentability which is characterized in that the gene according to Claim 1 or 2 on the chromosome is inactivated.

5. The yeast according to Claim 4, wherein the yeast belongs to Saccharomyces cerevisiae.

6. The yeast according to Claim 4 or 5, wherein the sequence at positions 4388 through 7885 in the nucleotide sequence represented by SEQ ID NO: 1 is disrupted.

7. Saccharomyces cerevisiae YHK1243 (FERM BP-5327).

8. Dough containing the yeast according to any of Claims 4-7.

9. A process for making bread which comprises adding the yeast according to any of Claims 4-7 to dough.

10. A process for producing ethanol which comprises culturing the yeast according to any of Claims 4-7 in a medium, allowing ethanol to accumulate in the culture, and recovering ethanol from the culture.

ABSTRACT

The present invention relates to a protein having the amino acid sequence represented by SEQ ID NO: 1, or a protein being capable of complementing the mutation exhibiting low-temperature-sensitive fermentability and having an amino acid sequence wherein one or more amino acid residues are added, deleted or substituted in the amino acid sequence represented by SEQ ID NO: 1; a gene which encodes said protein; and a gene which comprises DNA having the nucleotide sequence represented by SEQ ID NO: 1, or comprises DNA being capable of complementing the mutation exhibiting low-temperature-sensitive fermentability and having a nucleotide sequence wherein one or more DNAs are added, deleted or substituted in the nucleotide sequence represented by SEQ ID NO: 1. The present invention also relates to yeast belonging to the genus Saccharomyces and having low-temperature-sensitive fermentability which is characterized in that the above-mentioned gene on the chromosome is inactivated; dough containing said yeast; a process for making bread which comprises adding said yeast to dough; and a process for producing ethanol which comprises culturing said yeast in a medium, allowing ethanol to accumulate in the culture, and recovering ethanol from the culture.

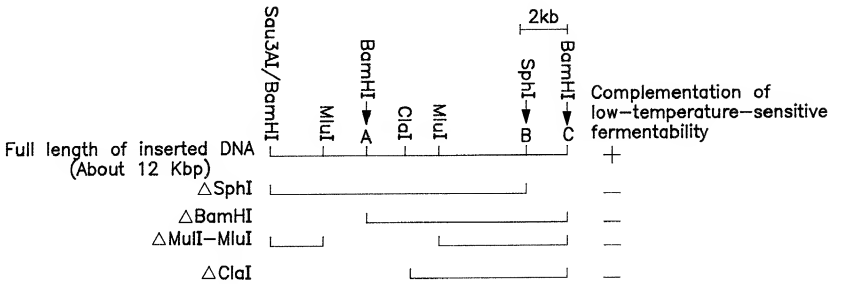
2kb
Sau3AI/BamHI
MluI
BamHI
A
ClaI
MluI
SphI
B
BamHI
C
Complementation of low-temperature-sensitive fermentability
Full length of inserted DNA (About 12 Kbp)
+
△SphI
—
△BamHI
—
△MluI—MluI
—
△ClaI
—

FIG. 1

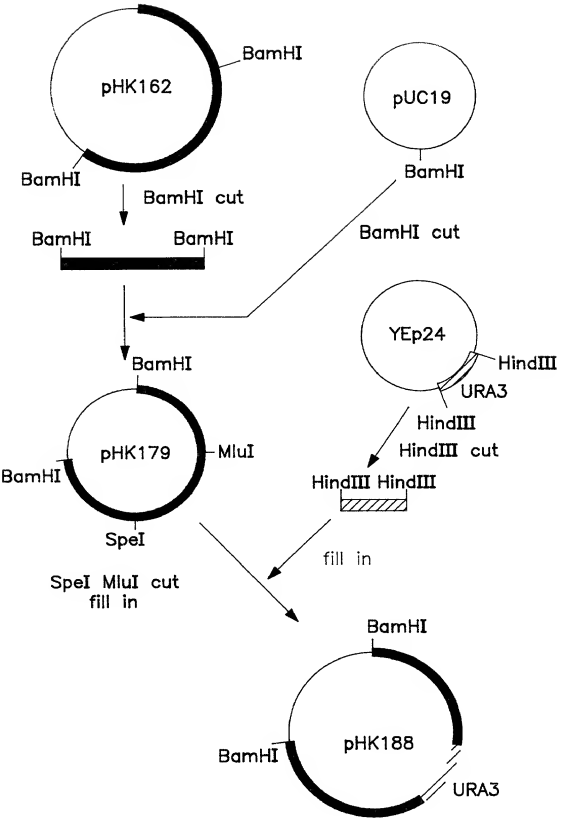
pHK162
BamHI
BamHI
BamHI cut
BamHI
BamHI
pUC19
BamHI
BamHI cut
YEp24
HindIII
URA3
HindIII
HindIII cut
HindIII HindIII
BamHI
pHK179
MluI
BamHI
SpeI
SpeI MluI cut
fill in
fill in
BamHI
pHK188
BamHI
URA3

FIG. 2

COMBI ED DECLARATION AND POWEI F ATTORNEY FOR 1 ATENT COOPERATION TREATY APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

NOVEL YEAST GENE

the specification of which was filed as PCT international application No. PCT/JP96/03862 on December 27, 1996 and was amended under PCT Article 19 on _____(if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35 U.S.C. §119(a)-(d) or §365(b), of any foreign application(s) for patent or inventor's certificate, or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate, or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Country	Application No.	Filed (Day/Mo./Yr.)	(Yes/No) Priority Claimed
JP	343700/95	28/12/95	Yes

I hereby appoint Joseph M. Fitzpatrick (Registration No. 17,398), Lawrence F. Scinto (Registration No. 18,973), William J. Brunet (Registration No. 20,452), Robert L. Baechtold (Registration No. 20,860), John A. O'Brien (Registration No. 24,367), John A. Krause (Registration No. 24,613), Henry J. Renk (Registration No. 25,499), Peter Saxon (Registration No. 24,947), Anthony M. Zupcic (Registration No. 27,276), Charles P. Baker (Registration No. 26,702), Stevan J. Bosses (Registration No. 22,291), Edward E. Vassallo (Registration No. 29,117), Ronald A. Clayton (Registration No. 26,718), Lawrence A. Stahl (Registration No. 30,110), Laura A. Bauer (Registration No. 29,767), Leonard P. Diana (Registration No. 29,296), David M. Quinlan (Registration No. 26,641), Nicholas N. Kallas (Registration No. 31,530), William M. Wannisky (Registration No. 28,373), Lawrence S. Perry (Registration No. 31,865), Robert H. Fischer (Registration No. 30,051), Christopher Philip Wrist (Registration No. 32,078), Gary M. Jacobs (Registration No. 28,861), Michael K. O'Neill (Registration No. 32,622), Bruce C. Haas (Registration No. 32,734), Scott K. Reed (Registration No. 32,433), Scott D. Malpede (Registration No. 32,533), Fredrick M. Zullow (Registration No. 32,452), Richard P. Bauer (Registration No. 31,588), Warren E. Olsen (Registration No. 27,290), Abigail F. Cousins (Registration No. 29,292), Steven E. Warner (Registration No. 33,326), Thomas J. O'Connell (Registration No. 33,202), Penina Wollman (Registration No. 30,816), David L. Schaeffer (Registration No. 32,716), Jack S. Cubert (Registration No. 24,245), Mark A. Williamson (Registration No. 33,628), John T. Whelan (Registration No. 32,448), Jean K. Dudek (Registration No. 30,938), Raymond R. Mandra (Registration No. 34,382), Dominick A. Conde (Registration Nn. 33,856), Steven C. Bauman (Registration No. 33,832), Pasquale A. Razzano (Reg. No. 25,512), John W. Behringer (Registration No. 23,086), Robert C. Kline (Registration No. 17,739), Mark J. Itri (Registration No. 36,171), William C. Hwang (Registration No. 36,169), Michael P. Sandonato (Registration No. 35,345), Jack M. Arnold (Registration No. 25,823), John D. Carlin (Registration No. 37,292), Daniel S. Glueck (Registration Nn. 37,838), Victor J. Geraci (Registration No. 38,157), Joseph W. Raguss (Registration No. 38,586), Brian L. Klock (Registration No. 36,570), Anne M. Maher (Registration No. 38,231), William J. Zak, Jr. (Registration No. 38,668), Thomas D. Pease (Registration No. 35,317), Bruce M. Wexler (Registration No. 35,409), and Robert S. Mayer (Registration No. 38,544) my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Address all correspondence to:

FITZPATRICK, CELLA, HARPER & SCINTO
277 Park Avenue
New York, N.Y. 10172
Telephone No. (212) 758-2400

COMBINED DECLARATION AND POWER OF ATTORNEY
FOR PATENT COOPERATION TREATY APPLICATION
(Page 2)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole or First Inventor Hideki Kawasaki
Inventor's Signature Hideki Kawasaki
Date July 18, 1997 Citizen/Subject of JAPAN
Residence Same as Post Office Address
Post Office Address 403-1-303, Arakawaoki-Nishi-ku 2-chome, Tsuchiura-shi, Ibaraki 300-11 Japan

Full Name of Second Joint Inventor, if any Masaya Tokai
Inventor's Signature Masaya Tokai
Date July 25, 1997 Citizen/Subject of JAPAN
Residence Same as Post Office Address
Post Office Address 3-5-9-209, Wakabayashi, Setagaya-ku, Tokyo 154 Japan

Full Name of Third Joint Inventor, if any Yasuhiro Kikuchi
Inventor's Signature Yasuhiro Kikuchi
Date July 18, 1997 Citizen/Subject of JAPAN
Residence Same as Post Office Address
Post Office Address 2-11-2-3-3, Matsushiro, Tsukuba-shi, Ibaraki 305 Japan

Full Name of Fourth Joint Inventor, if any Kozo Ouchi
Inventor's Signature Kozo Ouchi
Date July 30, 1997 Citizen/Subject of JAPAN
Residence Same as Post Office Address
Post Office Address 2-2-9, Sakuradai, Hasuda-shi, Saitama 349-01 Japan